Cyclic GMP Binding Activity in Dictyostelium discoideum

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CYCLIC GMP BINDING ACTIVITY IN *DICTYOSTELIUM DISCOIDEUM*

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1. Introduction

Cyclic AMP mediates cell aggregation in *Dictyostelium discoideum* by acting as chemoattractant [1] and as a signal transmitter [2–4]. Within 10 s after the application of cAMP, cells of *D. discoideum* build a pseudopod towards the attractant source [5] and about 60 s later release cAMP to the extracellular medium [3]. In addition to these short term effects, cAMP also has long term effects controlling the program for cell differentiation. Thus, the application of cAMP in pulses shorten the time interval between starvation and cell aggregation by speeding up the appearance of cell to cell contact sites A [6], cAMP binding sites [7–9] and cell surface bound phosphodiesterase [8,9]. All these components are essential for cell aggregation.

During the last year evidence has accumulated supporting a messenger function for cGMP during chemosensory transduction [7,10–12]. Within 2 s after the application of a cAMP signal the cellular cGMP content increases reaching a peak at 10 s and recovering pre-stimulation levels within 30 s [10]. In addition, all known attractants elevate the cGMP content of the specific species with similar kinetics [11–14]. These results agree with a messenger role for cGMP during the short and/or long term effects of cAMP in *D. discoideum*. However, the molecular mechanism controlling cGMP synthesis and action remains largely a puzzle. In the present paper evidence is given about the existence of an intracellular soluble cGMP receptor showing high affinity and specificity for this nucleotide in *D. discoideum*.

2. Materials and methods

Dictyostelium discoideum, NC-4(H), was used for all experiments. Cells were grown on a solid medium and harvested as in [15]. After harvesting, cells were suspended in 10 mM phosphate buffer (pH 6.0) and washed twice in the same buffer. The cell suspension was adjusted to 10^7 cells/ml and starved by shaking [16].

After shaking (2 h if not stated otherwise), cells were centrifuged, resuspended in 5 mM Tris–Cl (pH 7.5), and adjusted to 2×10^8 cells/ml. Cells were homogenized by freezing at −20°C and thawing at 0°C once under agitation followed by sonication for 5 s three times at 0°C using a Branson B12 sonifier with microtip at position 3. After sonication, the homogenate was centrifuged at 30 000 × *g* for 10 min (at 0°C in an SS-34 Sorvall rotor) and the supernatant was centrifuged once more at 48 000 × *g* for 60 min (at 0°C in an SS-34 Sorvall rotor). Reaction mixtures contained 52 mM phosphate buffer (pH 6.5), 2.4 mM $MgSO_4$, 1.2 mM dithiothreitol (DTT), which is a phosphodiesterase inhibitor in *D. discoideum* [17], 1×10^{-8} M [8-^{3}H]cGMP (Amersham, 21 Ci/mmol) and the 48 000 × *g* supernatant in total vol. 250 μl. Reactions were started by addition of 100 μl 48 000 × *g* supernatant and terminated after 5 min incubation at 0°C by filtering 200 μl through 0.45 μm filters (diameter 24 mm, Schleicher and Schüll) [18]. The filters were then washed twice with 4 ml ice-cold phosphate buffer (52 mM, pH 6.5) containing 2.4 mM $MgSO_4$ and 1.2 mM DTT. After filtration, the filters were dissolved

in 7 ml of a Cellosolve–toluene mixture [18] and counted. Unspecific binding (< 0.5%) was calculated by adding 10 μl 10^{-3} M cGMP to the incubation mixture. cGMP binding to cell homogenates was calculated as pmol cGMP bound/10^7 cells of the original cell suspension. All samples were assayed in triplicate immediately after centrifugation. cGMP, cAMP, GMP and ATP were from Boehringer. 5′-AMP was from Sigma.

Phosphodiesterase activity was assayed according to the procedure in [19] under the same conditions than those described for cGMP binding activity.

3. Results

Figure 1 shows the time course of cGMP binding to the 48 000 × *g* supernatant of *D. discoideum* homogenates. Preliminary experiments had shown that most, if not all, cGMP binding activity is present in this fraction. Furthermore fig.1 shows that no appreciable cGMP hydrolysis is detected under the present assay conditions.

Figure 2 shows the change in cGMP binding activity during cell differentiation to aggregation competence. A small but reproducible increase occurs during the first 2 h of differentiation. cGMP binding activity is constant from pH 5.5–6.5 decreasing slowly at higher pH values (fig.3). Scatchard plots of cGMP binding activity are linear with a dissociation constant (K_d) of 5–6 × 10^{-10} M and 2–3 × 10^3 receptors/cell (fig.3). Finally table 1 shows the effect of various nucleotides

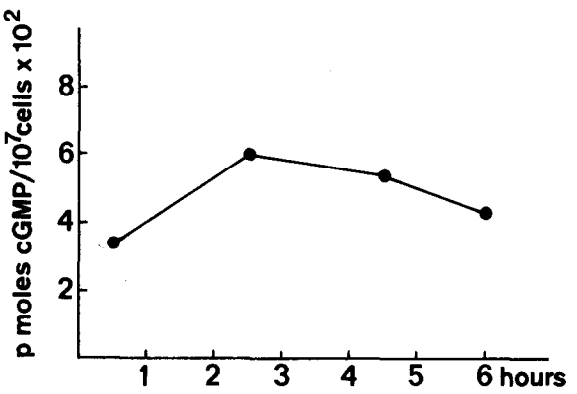

Fig.2. Changes in cGMP binding activity during cell differentiation to aggregation competence. Cell aggregation occurs about 5 h after the beginning of starvation. Concentration of cGMP, 1 × 10^{-8} M.

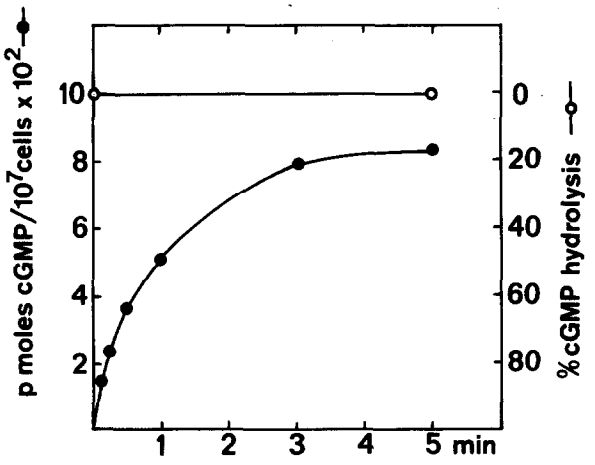

Fig.1. Time course of cGMP binding (–•–) by the 48 000 × *g* supernatant of *D. discoideum* homogenates. Hydrolysis of cGMP (–○–) under the same experimental conditions. Concentration of cGMP, 2 × 10^{-8} M.

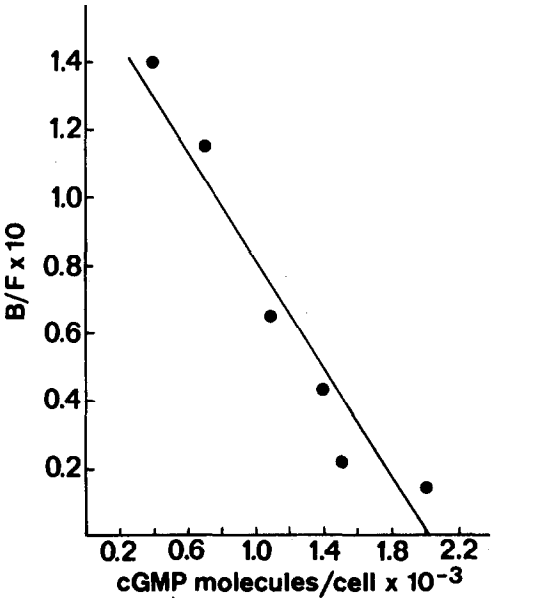

Fig.3. Scatchard plot of cGMP binding activity by the 48 000 × *g* supernatant of *D. discoideum*. Concentration range of cGMP = 0.25–10 × 10^{-9} M. B/F: the bound to free ratio of cGMP.

Table 1
Effect of various nucleotides on cGMP binding activity by the 48 000 × *g* supernatant of *D. discoideum*

Nucleotide added		% cGMP binding
None		(100)
cAMP	(10^{-5} M)	60
5'-AMP	(4×10^{-4} M)	101
ATP	(4×10^{-4} M)	98
GMP	(4×10^{-4} M)	94

All samples contained 1×10^{-8} M [8-^{3}H]cGMP plus the concentration of the nucleotide mentioned in the table. Results are calculated taking the binding of cGMP observed in the absence of other nucleotides as 100

on cGMP binding activity. Only cAMP at a concentration 10^3-fold higher than cGMP inhibits the binding of this nucleotide. ATP, 5'-AMP and 5'-GMP are without effect at a concentration of 4×10^{-4} M. Addition of 2 mM EGTA or 2 mM $CaCl_2$ has no effect on cGMP binding activity (data not shown).

4. Discussion

The present results indicate the existence of cGMP binding activity in the 48 000 × *g* supernatant of *D. discoideum*. This activity differs in its affinity and specificity from that in [20] for cAMP under similar conditions. The K_d for cGMP binding is about 10^4-fold lower than the Michaelian constant for cGMP hydrolysis by *D. discoideum* phosphodiesterases [12,21]. Furthermore, it is far more specific for cGMP than any of the various phosphodiesterases present in *D. discoideum* [12,21]. Therefore, it seems unlikely that the present cGMP binding activity represents the binding of cGMP to the catalytic site of a phosphodiesterase. Protein kinase activity in the 48 000 × *g* supernatant of *D. discoideum* is independent of the presence of cyclic nucleotides (unpublished). Developmentally regulated cAMP dependent protein kinases have been partially purified from *D. discoideum*, M-2 [22]. Although suggestive, these results need confirmation with the most commonly used species of *D. discoideum*, NC-4, and the axenic strain Ax.2. Purification of the present cGMP binding activity (in progress) will clarify whether this activity is associated to protein kinase, phosphodiesterase or any other type of receptor. Soluble tissue proteins, without definable enzymic activity, binding cGMP with high affinity have been isolated from various mammalian sources (reviewed [23]). Similar cGMP binding activity has been observed in cell homogenates of *D. mucoroides* and *Polysphondylium violaceum* (H. W., unpublished). The high affinity and specificity make the present cGMP binding activity an obvious candidate for mediating cGMP action in vivo in *D. discoideum*. The calculated number of cGMP receptors is however about 100-fold smaller than the maximal number of cGMP molecules generated in response to cAMP or folic acid [10,12]. Yet, theoretical calculations indicate that only 10^3 cAMP receptors are occupied when a cell reacts chemotactically [24]. In addition, the experiments [5], in which cells are stimulated with the help of a microcapillary, also indicate that the occupancy of a few receptors is enough to trigger a chemotactic response. Because in *D. discoideum*, NC-4, the number of cGMP molecules generated is similar to the number of cAMP receptors occupied [13] the calculated number of cGMP receptors/cell will be sufficient to mediate the transduction of a physiological cAMP signal.

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